AMENDMENT A

In addition to the foregoing, please enter the following amendments:

IN THE SPECIFICATION:

On page 5, please replace the last paragraph with the following:

The regulatory sequences can consist of an inducible promoter, in combination with an operator sequence. As used herein, the term "operator" or "operator sequence" refers to a polynucleotide sequence to which a repressor protein can bind, thereby regulating the expression of a gene. Any inducible promoter that is functional within Gram-negative bacterium can be used. It is preferred that the promoter or combination of the promoter and operator be strictly inducible so that there is little or no production of levansucrase in the absence of the inducing agent. In one preferred embodiment, the regulatory sequence is one that is functional in members of the genus *Agrobacterium*, and in particular *A. tumefaciens*. Examples of suitable regulator sequences include, but are not limited to, the P*lac* promoter and operator of *E. coli* (**SEQ. ID. NO. 1**), the *noc*R gene (**SEQ. ID. NO. 3**), which encodes for the transcriptional activator of Pi2 (*noc*) (**SEQ. ID. NO. 2**), and in the presence of the noc1 operon (**SEQ. ID. NO. 2**) which encodes for the nopaline transport system of *A. tumefaciens* (Von Lintig et al. (1991) *Molec. Plant Microbe Interaction*, 4:370-378) and the P_{BAD} promoter (**SEQ. ID. NO. 5**) and araC operator (**SEQ. ID. NO. 4**) of *E. coli* (Gallegos et al. (1997) *Microbiol. Mol. Biol. Rev.* 61:393-410).

a1

On page 7, please replace the first paragraph with the following:

Any method capable of introducing the construct into the genome of the bacterial vector can be use. In one embodiment, the construct is inserted by the use of homologous recombination in particular the method of Ruvkun and Ausubel ((1981) *Nature*, 289:85-88). In this method, a mutation, in the form of the recombinant construct of the present invention, is directed to a specific locus on the chromosome by homologous exchange recombination. Any locus which allows the inducible expression of levansucrase and does not impede with the DNA transfer machinery can be used. In one embodiment, the construct is inserted at the tetR/tetA loci (**SEQ. ID. NO. 9**) of *Agrobacterium* (Luo and Farrand (1999) *J. Bacteriol* 181:618-626).

a2

On page 9, please replace Example 2 with the following:

Example 2

Counter Selection by Genomic Incorporation of sacB

A construct was assembled that specifically targeted the sacB locus to the tetR/tetA loci (**SEQ. ID. NO. 9**) in *A. tumefaciens*. An *Eco*RI fragment from pSWE8.5 bearing the tetR/tetA loci (**SEQ. ID. NO. 9**) was subcloned into pGEM T-Easy. The resulting plasmid was digested with Hind III and a 0.5 kb fragment at the tetR locus was replaced with the 3.8 kb BamHI insert from pUM24 containing the nptI-sacB-sacR region (Reid and Collmer (1987) Gen 57:239-246). This step was accomplished by annealing after adding homopolymeric G and C tails to the vector and insert, respectively. The resulting construct was electroporated into *Agrobacterium* strain NT1/pEHA 105 and transformants were selected on kanamycin (50 mg/L) LB plates. Since the pGEM backbone was not expected to replicate in *A. tumefaciens*, kanamycin resistant transformants were presumed to be due to chromosomal integration of the nptI-sacB-sacR cassette at the tetR/tetA loci (**SEQ. ID. NO. 9**). Individual colonies were picked and replica plated to kanamycin supplemented LB medium with and without sucrose (3% w/v). The resulting bacterial patches that showed little or no growth on sucrose, but vigorous growth on kanamycin alone, were recovered, diluted and spread on kanamycin plates to isolate individual colonies. These were again tested as before until an isolate that consistently gave no growth on sucrose was recovered.

On pages 9-10, please replace Example 3 with the following:

Example 3

Use of the *E. coli* Plac/repressor System to Control sacB Expression

The *E. coli* lactose operon (**SEQ. ID. NO. 1**) is tightly regulated by the presence of a 21 bp operator that resides immediately down stream of the Plac promoter (**SEQ. ID. NO. 1**). In the absence of β-galactose sugar, the lac repressor will bind to the cis operator and prevent RNA polymerase initiation. In the presence of a β-galactose sugar, the lac repressor cannot bind to the operator and thus RNA polymerization proceeds. This system may be exploited as a strategy for tight regulation of the sacB expression in *Agrobacterium tumefaciens* cells.

The sacB open reading frame (ORF) can be subcloned downstream of the Plac promoter element (**SEQ. ID. NO. 1**) coupled with the 21 bp operator sequence. The lacI cassette (**SEQ. ID. NO. 6**) coding for the lac repressor may be ligated to the derived sacB cassette. The genetic element carrying the Plac-sacB and lac repressor cassettes can be introduced to the chromosome of *Agrobacterium tumefaciens* via homologous recombination. The preferred site for the recombination event would be the tetR/tetA loci (**SEQ. ID. NO. 9**) of *Agrobacterium* recently described by Luo and Farrand ((1999) *J. Bacteriol.* 181:618-626).

A3 Cont

This strategy will be useful for the genetic engineering of both monocotyledonous and dicotyledonous plant species. Various steps are followed in the *Agrobacterium*-mediated transformation of plant species. Generally the first step involves the inoculation of the explant (plant cells or tissue segments) with *Agrobacterium tumefaciens* cells. The explant can be leaf segment, cotyledon, stem, root, flower part or cells thereof. After a period of one to seven days, generally termed the co-cultivation period, the explant is transferred to plant regeneration medium supplemented with sucrose as the carbon source. The sacB system in this example may be induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) at levels ranging from 0.1 μM up to 1 mM and/or lactose at levels from 0.1 μM to 1 mM.

[On page 10, please replace Example 4 with the following:]

Example 4

<u>Use of the Nopaline-Inducible Marker System to Regulate sacB Expression</u>

The Pi2(noc) promoter (**SEQ. ID. NO. 2**) (Von Lintig et al. (1991) *Molec. Plant Microbe Interaction* 4:370-378) from *Agrobacterium tumefaciens* is induced in the presence of nopaline. This regulatory sequence may be employed as a strategy to regulate sacB expression in *Agrobacterium tumefaciens* cells. In this example, the sacB ORF will be subcloned down stream of the Pi 2(noc) promoter (**SEQ. ID. NO. 2**). The resultant cassettes preferably will be introduced into the chromosome of *Agrobacterium tumefaciens* via homologous recombination. The preferred site for recombination is the tetR/tetA loci (**SEQ. ID. NO. 9**). The nocR gene (**SEQ. ID. NO. 3**), which encodes for the transcriptional activator of Pi2(noc) (**SEQ. ID. NO. 2**), and the noc1 operon (**SEQ. ID. NO. 2**) which encodes for the nopaline transport system can be supplied by the vir region of the resident Ti plasmid, or by cloning these two loci onto a self-

replicating plasmid. In this example the sacB counter selection (suicide) system may be induced upon the addition of nopaline at levels ranging from 50 μg/L up to 200 μg/L.

On page 10-11, please replace Example 5 with the following:

B3 cont

Example 5

<u>Use of the *E. coli* araC Regulator to Control sacB Expression</u>

The *E. coli* P_{BAD} promoter (**SEQ. ID. NO. 5**) is highly induced in the presence of L-arabinose. The induction of the system is controlled by the presence of a cis acting element upstream of the P_{BAD} promoter (**SEQ. ID. NO. 5**), araC (**SEQ. ID. NO. 4**) (Gallegos et al. (1997) *Microbiol. Molec. Biol. Rev.* 61:393-410). This system can be utilized to regulate the expression of the sacB in *Agrobacterium tumefaciens* cells by placing the araC cis element (**SEQ. ID. NO. 4**) just 5' to the P_{BAD} promoter (**SEQ. ID. NO. 5**) (Luo and Farrand (1999) *J. Bacteriol.* 181:618-626); Newman and Fuqua (1999) *Gene* 227:197-203) and subsequently subcloning the sacB open reading frame downstream of the assembled DNA elements. In this example, counter selection of the bacterial cells can be induced following the co-cultivation period in plant transformation protocols by supplementing the regeneration medium with levels of L-arabinose ranging from 5 g/L up to 20 g/L.

On page 11, please replace Example 6 with the following:

Example 6

<u>Use of the traCDG Promoter to Control sacB Expression</u>

The sacB gene can be placed under the control of the *traCDG* promoter (**SEQ. ID. NO. 7**) (Farrand et al., (1996) Bacteriol. 178:4233-4247; Oger et al., (1998) Mol. Microbiol. 27:277-288; Luo and Farrand, (1999) Proc. Natl. Acad. Sci. USA 96:9009-9014). Initiation of this promoter is absolutely dependent upon activated TraR (**SEQ. ID. NO. 7**). Second, the TraR (**SEQ. ID. NO. 7**) will be placed under the direct control of an opine-responsive promoter system. We will fuse traR (**SEQ. ID. NO. 7**) directly to a fragment of DNA containing the *occ* promoter (**SEQ. ID. NO. °**) from the octopine-type Ti plasmid pTiR10. This promoter is activated by OccR (**SEQ. ID. NO. 8**), a lysR-like activator in response to the opine, octopine (Habeeb, et al., 1991). The *occR* gene (**SEQ. ID. NO. 8**) is located directly adjacent to the occ

promoter (**SEQ. ID. NO. 8**) and will be included in the recombinant construct. One can provide a copy of traM (**SEQ. ID. NO. 7**), which encodes for the antiactivator, in the traR-pOcc-occ construct. When the two constructs are combined in an *Agrobacterium* host, expression of *sac*B should be strongly suppressed in the absence of the opine. However, addition of octopine (which will activate expression of traR (**SEQ. ID. NO. 7**) leading to accumulation of the activator to levels that overcome the antiactivator, TraM (**SEQ. ID. NO. 7**)) should strongly induce *sacB*.

B3 cont.

The genetic elements described above can be introduced to a neutral site in the chromosome of *Agrobacterium tumefaciens*, with respect to plant transformation effects, via homologous recombination. The preferred site for the recombination event would be the tetR/tetA loci (**SEQ. ID. NO. 9**) of *Agrobacterium*. However, with the imminent availability of the genome sequence of *Agrobacterium* strain C58 one should be able to identify alternative sites within the chromosome.

On page 11-12, please replace Example 7 with the following:

Example 7

Use of a Second Copy of the sacB Gene

To circumvent potential mutational inactivation of sacB a second copy of the gene can be provided. To prevent recombination between the two copies one can use a sacB gene from another bacterium. Possibilities of alternative *sacB* sources include *B. stearothermophilus*, *B. amyloliquefaciens*, or *Streptococcus mutans*. The sequence for each of these is available in the data bases. The alternative *sacB* gene can be fused to a second copy of the TraR-dependent traCDG promoter (**SEQ. ID. NO. 7**).